

**CERTIFICATE OF MAILING BY "EXPRESS MAIL"**

**"Express Mail" Mailing Label No.** 154556307US

Date of Deposit December 30, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

JINNY NORMAN  
(Typed or Printed Name of Person Mailing Paper or Fee)

(Typed or Printed Name of Person Mailing Paper or Fee)

(Signature of Person Mailing Paper or Fee)

(Signature of Person Mailing Paper or Fee)

## CD40 BINDING COMPOSITIONS AND METHODS OF USING SAME

This invention was made with government support under Grant No. CA61348, awarded by the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

## Field of the Invention

10           This invention relates to a novel protein which  
binds to the intracellular region of the CD40 receptor.

## Background of the Invention

CD40 antigen is a cell surface transmembrane 45-kDa glycoprotein receptor expressed on a number of cell types, including B-lymphocytes ("B cells"). Stamenkovic et al. (1989) EMBO J. 8:1403-1410. It is a member of the tumor necrosis factor receptor family and, like other members, it appears to possess no intrinsic signaling capacity (e.g., kinase activity), suggesting that signal transduction is likely mediated by associating molecules. CD40 antigen has a short cytoplasmic tail (65 amino acid residues), and mutagenesis studies suggest that Thr<sup>234</sup> in the cytoplasmic domain is essential for signal transduction. Inui et al. (1990) Eur. J. Immunol. 20:1747-1753.

The ligand to CD40, "CD40L", is expressed on activated T-helper cells. Armitage et al. (1992) Nature 357:80-82. Activation of CD40 receptor is critical for B-cell proliferation, cytokine production, immunoglobulin

Ins AI

class switching, and rescue of germinal center B cells from apoptosis following somatic mutation. Banchereau et al. (1991) Science 251:70-72; Liu et al. (1989) Nature 342:929-931; and Zhang et al. (1991) J. Immunol.

5 146:1836-1842.

Mutations in CD40L result in an immuno-deficiency (X-linked hyper-IgM syndrome) characterized by IgM-producing B cells that do not form germinal centers in response to foreign antigens. Allen et al. (1993) 10- Science 259:990-993; Korthauer (1993) Nature 361:539-541; and Fuleihan (1993) Proc. Natl. Acad. Sci., U.S.A. 90:2170-2173. Hyper-IgM syndrome is a rare disorder characterized by recurrent infections and is associated with low serum levels of IgG, IgA, and IgE, and normal or 15 increased levels of IgM. Clinical features of this syndrome include recurrent bacterial infections of the upper and lower respiratory tract, usually beginning in the first or second year of life. Ochs et al. (1993) Curr. Opin. Pediatr. 5:684-691. Pneumocystis carinii 20 pneumonia in early infancy, neutropenia, thrombocytopenia, hemolytic anemia, nephritis and arthritis also have been associated with this genetic disorder.

Activation and transduction through the CD40 25 pathway is in large part, responsible for B cell activation and accordingly, the cellular immune response. However, it is still unknown how the receptor transduces its signal. Thus, in view of the variety of immune responses mediated through the CD40 receptor, it would be 30 desirable to have a means to study the CD40 receptor pathway as well as modulate its effects. This invention satisfies this need and provides related advantages as well.

### Summary of the Invention

This invention provides a novel purified mammalian protein designated CD40bp having the ability to bind the cytoplasmic region or domain of a CD40 receptor.

Also provided by this invention are nucleic acid molecules that encode the mammalian protein which binds the intracellular domain of CD40.

An antibody, such as a monoclonal antibody, which specifically binds CD40bp is further provided by this invention.

Methods of using the proteins, nucleic acids and antibodies described above are further provided herein.

### Brief Description of the Figures

Figure 1 shows CD40bp interactions with hybrid proteins. Yeast transformants harboring CD40bp fused to the activation domain of GAL4 and the indicated expression plasmids encoding proteins fused to the DNA-binding domain of GAL4 were assayed in duplicate for  $\beta$ -galactosidase activity.

Figures 2A and 2B show the interaction of *in vitro* translated CD40bp with GST fusion proteins. [ $^{35}$ S]Methionine-labeled CD40bp or luciferase protein as control was incubated with GST alone, GSTCD40T (native CD40 cytoplasmic domain), or GSTCD40A (mutant CD40 cytoplasmic domain Thr<sup>234</sup>  $\rightarrow$  Ala). Following incubation and washing, GST beads were boiled in SDS-sample buffer and resolved on a 10% acrylamide gel, and bound protein was visualized by autoradiography. The left panel shows the signal from 5  $\mu$ l of labeled translated protein prior to incubation with GST beads.

Figures 3A through 3E show the association of CD40 and CD40bp *in vivo* in transfected 293T and BJAB

cells. In Figure 3A, 293T cells were cotransfected with HA epitope-tagged CD40bp and with vector, mutant CD40 (CD40A), or native CD40 (CD40T) expression constructs, metabolically labeled with [<sup>35</sup>S]methionine and

5 [<sup>35</sup>S]cysteine, and cell lysates analyzed by immunoprecipitation with an anti-CD40 monoclonal antibody. Figure 3B shows that immune complexes from the native CD40T-transfected cells were dissociated and re-immunoprecipitated with control antibody ( $\alpha$ -TSP), anti-

10 CD40 ( $\alpha$ CD40), or anti-HA tag ( $\alpha$ HA), which should recognize HA-tagged CD40bp. Figure 3C shows anti-CD40 immune complexes from transfected BJAB cells were either analyzed intact ( $\alpha$ -CD40) or dissociated and reimmunoprecipitated with an anti-HA tag antibody ( $\alpha$ -

15 CD40/ $\alpha$ -HA) or isotype-matched control antibody ( $\alpha$ -CD40/Control Ig). Five-fold more cell lysate was used for the double immunoprecipitations. Figure 3D is a northern blot analysis for CD40bp transcript expression in the SKW6.4 B-cell line. Figure 3E is a survey of

20 CD40bp transcript expression by RT-PCR. RNA from the indicated CD40-positive B-cell lines (B) and CD40-negative cell lines (T, T-cell line; E, epithelial cell line) was subjected to RT-PCR using CD40bp-specific oligonucleotide primers.

25 Figures 4A through 4E show the amino acid sequence and subsequent analysis of the CD40 binding protein. Figure 4A is the amino acid sequence of CD40bp (also Seq. ID. No. 2). The first underlined segment is the RING finger domain; Cys/His residues that are

30 invariant with respect to other proteins (shown in Figure 4B) are indicated in **bold**. These amino acid sequences also are listed as: Seq. ID. No. 3 (CD40bp); Seq. ID. No. 4 (TRAF2); Seq. ID. No. 5 (RAG1); Seq. ID. No. 6 (RING1); Seq. ID. No. 7 (52kd RNP); Seq. ID. No. 8 (UVS-2); and

35 Seq. ID. No. 9 (DG17). The second underlined region represents the coiled-coil domain (shown in Figure 4D).

The Cys/His residues between the RING finger and coiled-coil domains are marked by asterisks. Features of the CD40bp sequence are summarized schematically in Figure 4C. Homology within the C-terminal TRAF domains of the indicated proteins is shown in Figure 4E (also Seq. ID Nos. 10 through 12).

Figure 5 shows a nucleic acid sequence coding for full length CD40bp (also Seq. ID. No. 1). The initiation codon starts at nucleotide 211. The coding sequence ends at nucleotide 1911. The corresponding encoded amino acid sequence is shown in Seq. ID. No. 2.

### Detailed Description of the Invention

#### 15 Proteins and Polypeptides

This invention provides purified proteins having the ability to bind the cytoplasmic region of the CD40 receptor. Previous attempts using traditional methods, including co-immunoprecipitation and chemical cross-linking, have failed to identify molecules associating with the cytoplasmic domain of the CD40 receptor. Thus, Applicants are the first to provide such molecules. The purified proteins of this invention, termed "CD40bp" are defined by their specific ability to bind to the cytoplasmic domain of the CD40 receptor. The CD40 receptor is present on various cell types, including for example, B cells, dendritic cells, epithelial cells, monocytes, blood mononuclear cells, and some carcinoma cell lines. Any cell expressing CD40 is intended to be encompassed by the term "CD40+ cell". See Banchereau et al. (1991) Science 251:70-72; Caux et al. (1994) J. Exp. Med. 180:1263-1272; Fuleihan et al. (1993) Proc. Natl. Acad. Sci. 90:2170-2173; Werner-Favre et al. (1994) Immunology 81:111-114; and Stamenkovic et al. (1989) Embo J. 8:1403-1410.

In one embodiment of this invention, a purified protein is a human protein having an apparent molecular weight of about 64kD as determined by an SDS polyacrylamide gel under reducing conditions. In a  
5 separate embodiment, a protein has the amino acid sequence shown in Seq. ID. No. 2 and Figure 4A. Also provided by this invention are polypeptide fragments of the mammalian protein, the human 64kD protein or the protein having the amino acid sequence shown in Seq. ID.  
10- No. 2 and Figure 4E, each defined by the ability to bind to the cytoplasmic domain of the CD40 receptor using, for example, the *in vitro* binding assay described in Experiment II.

It is understood that functional equivalents of  
15 the protein shown in Figures 4A, the 64kD purified protein, or the polypeptide fragments thereof, e.g., as shown in Figures 4B or 4EE, and equivalents thereof, also are within the scope of this invention. One such equivalent includes chemical structures other than amino  
20 acids which functionally mimic the binding of the CD40bp to the cytoplasmic domain of the CD40 receptor ("muteins"). An additional example of an equivalent is a protein or polypeptide containing a distinct protein or polypeptide joined to CD40bp or its equivalent which  
25 varies the primary sequence of protein of this invention from the sequences provided in Figures 4A or 4E without necessarily affecting the binding of the resultant polypeptide or protein to the cytoplasmic domain of CD40. Where specific amino acids or other structures or  
30 sequences beyond the sequence shown in Seq. ID. No. 2 are presented, it is intended that various modifications which do not destroy the function of the binding site are within the definition of the proteins encompassed by this invention. For the purposes of this invention, the term  
35 "CD40bp" is intended to mean all of the proteins,

-7-

polypeptides, fragments and equivalents thereof, having the ability to bind the cytoplasmic domain of CD40.

An agent having the ability to inhibit the ability of CD40bp to bind to the cytoplasmic domain of CD40 receptor is further provided by this invention. Such agents include, but are not limited to, an anti-CD40bp antibody, a dominant inhibitory fragment of CD40bp or a soluble intracellular CD40. "Soluble intracellular CD40" is an intracellular portion of the CD40 receptor which binds CD40bp. These soluble receptors can be produced using the sequence of the cytoplasmic domain provided in Stamenkovic et al. (1989) supra and methods well known to those of skill in the art.

The terms "proteins" and "polypeptides" also are intended to include molecules containing amino acids linearly coupled through peptide bonds. As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the  $\alpha$ -amino group of another amino acid. Such polypeptides also can contain amino acid derivatives or non-amino acid moieties. The amino acids can be in the L or D form so long as the binding function of the polypeptide is maintained. The term amino acid refers both to the naturally occurring amino acids and their derivatives, such as TyrMe and PheCl, as well as other moieties characterized by the presence of both an available carboxyl group and an amine group. Non-amino acid moieties which can be contained in such polypeptides include, for example, amino acid mimicking structures. Mimicking structures are those structures which exhibit substantially the same spatial arrangement of functional groups as amino acids but do not necessarily have both the  $\alpha$ -amino and  $\alpha$ -carboxyl groups characteristic of amino acids.

As used herein, the term "hydrophobic" is intended to include those amino acids, amino acid

derivatives, amino acid mimics and chemical moieties which are non-polar. Hydrophobic amino acids include Phe, Val, Trp, Ile and Leu. As used herein, the term "positively charged amino acid" refers to those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are positively charged. Positively charged amino acids include, for example, Lys, Arg and His.

The proteins and polypeptides of this invention are distinct from native or naturally occurring proteins or polypeptides because they exist in a purified state. As used herein, the term "purified" when referring to a protein or a polypeptide or any of the intended variations as described herein shall mean that the compound or molecule is substantially free of contaminants normally associated with a native or natural environment.

The proteins and polypeptides of this invention can be obtained by a number of methods well known to those of skill in the art, which include purification, chemical synthesis and recombinant methods. For example, the proteins and polypeptides can be purified from CD40<sup>+</sup> cell or tissue lysates using methods such as immuno-precipitation with anti-CD40bp antibody, and standard techniques such as gel filtration, ion-exchange, reversed-phase, and affinity chromatography using a CD40 fusion protein as shown herein. For such methodology, see for example Deutscher et al., Guide to Protein Purification: Methods in Enzymology (1990) Vol. 182, Academic Press.

The proteins and polypeptides also can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA and the amino acid sequence provided in Figure 4A. The material so synthesized can



be precipitated and further purified, for example by high performance liquid chromatography (HPLC).

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual 2d ed. (Cold Spring Harbor Laboratory (1989)) using the host vector systems described and exemplified below. As an example, CD40bp fusion protein can be made by first utilizing a CD40<sup>-</sup> cell line such as 293T cells. The cells are first transiently transfected with pHTagCD40bp (constructed as described below). About 72 to 96 hours after transfection, the cells are lysed in 50mMTris Ph7.6 + 1% NP-40. CD40bp fusion protein is purified from the cell-extract using standard immunochemical means since it contains an hemagglutinin epitope tag allowing one to use commercially available anti-HA monoclonal antibody to purify the tagged molecule.

The CD40b protein and polypeptides have several utilities. For example, they can be bound to a column and used for the purification of CD40 receptors or to detect CD40 in a cell or tissue sample. They also are useful as immunogens for the production of anti-CD40bp antibodies as described below. They have further utility in an *in vitro* assay system to screen for immunosuppressant drugs and to test possible therapies.

When used to detect CD40, the CD40bp can be bound to a solid phase carrier for example, glass, polystyrene, polyethylene, dextran, nylon, natural and modified celluloses, polyacrylamides, glutathione-agarose beads and agaroses. Those skilled in the art will know of other suitable carriers for this purpose.

Accordingly, this invention also provides a method of detecting CD40 in a cell sample by first immobilizing CD40bp onto a solid support such as glutathione-agarose beads at a suitable concentration, eg., between about 5

-10-

mg/ml to about 12 mg/ml, and more preferably between about 6 mg/ml and about 10 mg/ml. The sample containing or suspected of containing CD40 is prepared and contacted with the beads under conditions favoring binding between the CD40 receptor and CD40bp. Suitable conditions are for example, those set forth in Experiment II. The beads are then subjected to conditions to release the complex from the solid support and protein complex can then be visualized by autoradiography.

10 The proteins of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers as defined below, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant which is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one.

15

20 However, for the purpose of illustration only, suitable adjuvants include, but are not limited to Freund's Complete and Incomplete, mineral salts and polynucleotides.

## 25 Nucleic Acids

Isolated nucleic acid molecules which encode amino acid sequences corresponding to CD40bp protein, mutein, antibodies and active fragments thereof are further provided by this invention. As used herein, "nucleic acid" shall mean single and double stranded DNA, cDNA and RNA, including anti-sense RNA. One can obtain an anti-sense RNA using the sequence provided in Figure 5 and the methodology described in Vander Krol et al.

30

35 (1988) BioTechniques 6:958. "Isolated" means separated

from other cellular components normally associated with DNA or RNA intracellularly.

In one aspect of this invention, the nucleic acid molecule encoding CD40bp protein or polypeptide has the sequence or parts thereof shown in Figure 5.

The invention also encompasses nucleic acid molecules which differ from that of the nucleic acid molecules shown in Figure 5, but which produce the same phenotypic effect. These altered, but phenotypically equivalent nucleic acid molecules are referred to "equivalent nucleic acids." Examples of such "equivalent nucleic acids" are those molecules which have a sequence which is homologous to sequence of Figure 5 and preferably have a homology of greater than about 50%, more preferably in excess of 90%. A homology of about 99% is most preferred. This invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule described hereinabove. This invention further encompasses nucleic acid molecules which hybridize to the nucleic acid molecule of the subject invention.

The nucleic acid molecules of this invention can be isolated using the technique described in Experiment I or replicated using PCR (Perkin-Elmer) and the methods described in Experiment III. For example, the sequence can be chemically replicated using PCR (Perkin-Elmer) which in combination with the synthesis of oligonucleotides, allows easy reproduction of DNA sequences. A DNA segment of up to approximately 6000 base pairs in length can be amplified exponentially starting from as little as a single gene copy by means of PCR. In this technique, a denatured DNA sample is incubated with two oligonucleotide primers that direct the DNA polymerase-dependent synthesis of new

-12-

complementary strands. Multiple cycles of synthesis each afford an approximate doubling of the amount of target sequence. Each cycle is controlled by varying the temperature to permit denaturation of the DNA strands, annealing the primers, and synthesizing new DNA strands. The use of a thermostable DNA polymerase eliminates the necessity of adding new enzyme for each cycle, thus permitting fully automated DNA amplification. Twenty-five amplification cycles increase the amount of target sequence by approximately  $10^6$ -fold. The PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202. Alternatively, one of skill in the art can use the sequence provided herein and a commercial DNA synthesizer to replicate the DNA. RNA can be obtained by using the isolated DNA and inserting it into a suitable cell where it is transcribed into RNA. The RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook et al. (1989) supra.

The invention further provides the isolated nucleic acid molecule operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off the nucleic acid molecule. Examples of such promoters are SP6, T4 and T7. Vectors which contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well known in the art. See for example, Gacesa and Ramji, Vectors: Essential Data Series (1994) John Wiley & Sons, N.Y., which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession

-13-

numbers for various suitable vectors. Preferable, these vectors are capable of transcribing RNA *in vitro* or *in vivo*.

5 Fragments of the sequence shown in Figure 5 and its equivalents are useful as probes to identify transcripts of the protein which may or may not be present. These nucleic acid fragments can be prepared, for example, by restriction enzyme digestion of the nucleic acid molecule of Figure 5 and then labeled with a  
10- detectable marker such as a radioisotope using well known methods. Alternatively, random fragments can be generated using nick translation of the molecule. For methodology for the preparation and labeling of such fragments, see Sambrook et al., Molecular Cloning: A  
15 Laboratory Manual Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989) supra. Nucleic acid fragments of at least 10 nucleotides are useful as hybridization probes. Isolated nucleic acid fragments also are useful to generate novel peptides. These peptides, in turn, are  
20 useful as immunogens for the generation of polyclonal and monoclonal antibodies.

As noted above, an isolated nucleic acid molecule of this invention can be operatively linked to a promoter of RNA transcription. These nucleic acid  
25 molecules are useful for the recombinant production of CD40bp proteins and polypeptides or as vectors for use in gene therapy. Accordingly, this invention also provides a vector having inserted therein an isolated nucleic acid molecule described above, for example, a viral vector,  
30 such as bacteriophages, baculoviruses and retroviruses, or cosmids, plasmids and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction  
35 enzyme to create complementary ends on both molecules that base pair with each other and which are then joined

together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the insert DNA that correspond to a restriction site in the vector DNA, which is then digested with a restriction enzyme that

5 recognizes a particular nucleotide sequence.

Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such

10 as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV) for high levels of transcription; transcription termination and RNA processing signals from

15 SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and anti-sense RNA.

20 An additional example of a vector construct of this invention is a bacterial expression vector including a promoter such as the lac promoter and for transcription initiation, the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al., (1989) supra). Similarly, a

25 eucaryotic expression vector is a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the

30 sequences described in methods noted above.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce CD40bp proteins and polypeptides. It is implied that these expression vectors must be replicable in the host

35 organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include

viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues *in vivo* because of their high levels of

5 expression and efficient transformation of cells both *in vitro* and *in vivo*. When a nucleic acid is inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic cell and the host cell replicates, CD40bp can be

10 recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well known methods. See Sambrook et al., (1989) supra. In addition

15 to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic acid can be inserted into the host cell by methods well known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See

20 Sambrook et al. (1989) supra for this methodology. Thus, this invention also provides a host cell, e.g. a mammalian cell, a animal cell, a human cell, or a bacterial cell, containing a nucleic acid molecule encoding a CD40bp protein or polypeptide.

25 Using the host vector system described above, a method of producing recombinant CD40bp or active fragments thereof is provided by growing the host cells described herein under suitable conditions such that the nucleic acid encoding the CD40 protein or polypeptide is

30 expressed. Suitable conditions can be determined using methods well known to those of skill in the art, see for example, Sambrook et al., (1989) supra. Proteins and polypeptides purified from the cellular extract and thereby produced in this manner also are provided by this

35 invention.

-16-

A vector containing the isolated nucleic acid encoding CD40bp also is useful for gene therapy to modulate CD40<sup>+</sup> cellular functions such as CD40-regulated antibody production and immune disorders caused by CD40 disfunction. The terms "CD40<sup>+</sup> cellular function" is intended to mean cellular functions which are affected by the binding of the receptor to its ligands, i.e., CD40L and CD40bp, alone or in combination with each other. In some instances, it is desirable to augment CD40<sup>+</sup> function to increase production of antibodies by introducing into the cell CD40bp protein or nucleic acid. A related CD40 immune disfunction wherein CD40 function is suitably augmented is Hyper-IgM Syndrome. In other instances, it is desirable to down-regulate CD40<sup>+</sup> cellular function by introducing into the cell a CD40bp antibody or a nucleic acid encoding an anti-CD40bp antibody or alternatively, a CD40bp fragment or nucleic acid encoding it which is a dominant negative inhibitor of functionally intact native CD40bp. This therapy will inhibit or disable CD40 signaling and therefore is a useful therapy where constitutive, unabated activation of B cells leads to production of inordinate amounts of antibodies contributing to an autoimmune disease or state.

When used for gene therapy, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral vector. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al., (1989) BioTechniques 7:980-990).



The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, et al. (1989), PNAS USA 86:8912; Bordignon, (1989), PNAS USA 86:8912-52; Culver, K., (1991), PNAS USA 88:3155; and Rill, D.R. (1991), Blood 79(10):2694-700. Clinical investigations have shown that there are few or no adverse effects associated with the viral vectors, see Anderson, (1992), Science 256:808-13.

10-

### Antibodies

Also provided by this invention is an antibody capable of specifically forming a complex with CD40bp protein or a fragment thereof, as well as nucleic acids encoding them. Vectors and host cells containing these nucleic acids also are encompassed by this invention. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, rabbit or human antibodies.

As used herein, an "antibody or polyclonal antibody" means a protein that is produced in response to immunization with an antigen or receptor. The term "monoclonal antibody" means an immunoglobulin derived from a single clone of cells. All monoclonal antibodies derived from the clone are chemically and structurally identical, and specific for a single antigenic determinant. The hybridoma cell lines producing the monoclonal antibodies also are within the scope of this invention.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies, as well as deducing their corresponding nucleic acid sequences, are known in the art, see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988) and Sambrook et al. (1989) supra. The monoclonal

-18-

antibodies of this invention can be biologically produced by introducing CD40bp or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with  
5 myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas. Accordingly, the hybridoma cells producing the monoclonal antibodies of this invention also are provided.

Thus, using the CD40bp protein or fragment  
10- thereof, and well known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind CD40bp.

If a monoclonal antibody being tested binds -  
15 with CD40bp, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this invention  
20 by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding CD40bp with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the invention as shown by a  
25 decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with CD40bp with which it is normally reactive,  
30 and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this  
35 invention.

-19-

The term "antibody" also is intended to include antibodies of a different isotype than the monoclonal antibody of this invention. Particular isotypes of a monoclonal antibody can be prepared either directly by  
5 selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described in Steplewski et al. (1985)  
10- Proc. Natl. Acad. Sci. 82:8653 or Spira et al. (1984) J. Immunol. Methods 74:307. Thus, the monoclonal antibodies of this invention would include class-switch variants having specificity for an epitope on CD40bp.

This invention also provides biological active  
15 fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody fragments can include, but are not limited to:

20 (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

25 (2) Fab', the fragment of an antibody molecule obtained by treating with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

30 (3) (Fab')<sub>2</sub>, the fragment of the antibody that is obtained by treating with the enzyme pepsin without subsequent reduction; F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds;

35 (4) Fv, defined as a genetically engineered fragment containing the variable region of the light

-20-

chain and the variable region of the heavy chain expressed as two chains; and

- (5) SCA, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

A specific examples of "biologically active antibody fragment" include the CDR regions of the antibodies. Methods of making these fragments are known in the art, see for example, Harlow and Lane, (1988) supra.

The antibodies of this invention also can be modified to create chimeric antibodies (Oi, et al. (1986) BioTechniques 4(3):214). Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, et al., Science, 232:100, 1986). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, it is responsible for the specificity of the antibody. The anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The animal immunized will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the second

-21-

animal, which are specific for the monoclonal antibodies produced by a single hybridoma which was used to immunize the second animal, it is now possible to identify other clones with the same idiotypic as the antibody of the hybridoma used for immunization.

Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

As used in this invention, the term "epitope" is meant to include any determinant capable of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Also encompassed by this invention are proteins or polypeptides that have been recombinantly produced, biochemically synthesized, chemically synthesized or chemically modified, that retain the ability to bind CD40bp or a fragment thereof, as the corresponding native polyclonal or monoclonal antibody.

-22-

The antibodies of this invention can be linked to a detectable agent or a hapten. The complex is useful to detect the CD40bp protein and fragments in a sample using standard immunochemical techniques such as

5 immunohistochemistry as described by Harlow and Lane (1988) supra. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays

10- are the enzyme linked immunoassay (ELISA) radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of CD40bp using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward,

15 reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

20 Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin,

25 which reacts avidin, or dinitropherryl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies. See Harlow and Lane (1988) supra.

The monoclonal antibodies of the invention can be bound to many different carriers. Examples of well-

30 known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention.

35 Those skilled in the art will know of other suitable

-23-

carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art.

5 Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable  
10 labels for binding to the monoclonal antibody, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody of the invention can be done using standard techniques common to those of ordinary skill in  
15 the art.

For purposes of the invention, CD40bp may be detected by the monoclonal antibodies of the invention when present in biological fluids and tissues. Any sample of CD40<sup>+</sup> cell or tissue lysate containing a  
20 detectable amount of CD40bp can be used.

### Compositions

This invention also provides compositions  
25 containing any of the above-mentioned proteins, muteins, polypeptides or fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for administration.  
30 As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The  
35 compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and

adjuvants, see Martin, Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton (1975)). These compositions can be used for the preparation of medicaments for the diagnosis and treatment of pathologies associated with the loss of functional CD40bp.

### Utilities

The antibodies and nucleic acid molecules of this invention also are useful to detect and determine the presence of CD40bp in a cell or a sample taken from a patient. Because the presence of CD40bp in a cell is an important indicator of immune function and CD40 disfunction; the absence of CD40bp has been implicated in a number of immunological diseases, such as systemic lupus. It is therefore advantageous to use the antibody to screen for the presence or absence of CD40bp in a CD40<sup>+</sup> or CD40<sup>-</sup> tissue sample cell extract taken from a subject. This procedure is preferred over the use of hybridization assays to detect CD40bp transcript levels because it is a precise indicator of loss of CD40bp in the cells. That is, CD40bp transcript may be present in the cell but not translated thereby leading to the CD40bp deficiency and immune disfunction.

CD40bp also is useful to detect the presence of CD40 in a cell or tissue sample suspected of containing the receptor. The sample is prepared using methods well known in the art (see, for example, Armitage et al. (1992) Nature 357:80-82; Armitage et al. (1993) Eur. J. Immunol. 23:2326-2331; Caux et al. (1994) J. Exp. Med. 180:1263-1272; Torres and Clark (1992) J. of Immunol. 148(2):620-626; and Werner-Favre, et al. (1994) 81:111-114). A CD40bp or polypeptide is then added to the sample under conditions favoring binding of the protein to the receptor for example, as provided in Example II.



The use of the compositions and methods in vitro provides a powerful bioassay for screening for drugs which are agonists or antagonists of CD40 pathway function in these cells. It also provides a powerful assay to determine whether an agent of interest, such as a pharmaceutical, is useful to treat a CD40 related disorder or to further augment CD40 function. For example, the composition to be tested can be added prior to, simultaneously or subsequent to CD40bp as described above. A separate "control" assay is run simultaneously under the same conditions but without the addition of the composition or drug being tested. If the agent inhibits binding of CD40 to CD40bp (as compared to control) the agent is a candidate for immunosuppressive therapy. If the agent augments binding, then the agent is a candidate for immunotherapy for conditions such as hyper-IgM syndrome.

Accordingly, this invention also provides a method for screening for a CD40 immunosuppressive agent, comprising the steps of: a) providing a CD40 cytoplasmic domain receptor bound to a solid support; b) contacting the agent with the receptor bound support of step a) under conditions favoring binding of the cytoplasmic domain to the receptor to CD40bp; c) contacting detectably-labeled CD40bp to the solid support of step b) under conditions favoring binding of CD40 cytoplasmic domain receptor to CD40bp; d) detecting the presence of any complex formed between CD40 receptor and CD40bp to form CD40 receptor-CD40bp complex; and e) the absence of CD40 receptor-CD40bp complex being indicative that the agent inhibits binding of CD40bp to CD40 receptor and therefore is an immunosuppressive agent.

This invention provides an alternative method for screening for a CD40 immunosuppressive agent, which comprises the steps of a) providing a CD40 cytoplasmic domain receptor bound to a solid support; b) contacting

-26-

detectably-labeled CD40bp to the solid support of step a) under conditions favoring binding of CD40 cytoplasmic domain receptor to CD40bp; c) contacting the agent to be screened with the receptor bound support of step b) under  
5 conditions favoring binding of the cytoplasmic domain to the receptor to CD40bp; d) detecting the presence of any complex formed between CD40 receptor and CD40bp to form CD40 receptor-CD40bp complex; and e) the absence of CD40 receptor-CD40bp complex being indicative that the agent  
10- inhibits binding of CD40bp to CD40 receptor and therefore is an immunosuppressive agent.

As is apparent to those of skill in the art, the above compositions can be combined with instructions for use to provide a kit for a commercially available  
15 screen.

The compositions provided herein also are useful to modulate the CD40 receptor pathway and cellular functions associated with this pathway, for example, CD40-related cytokine production, B cell proliferation,  
20 and hyper-IgM syndrome. Additional CD40-related functions are known to those of skill in the art. (See, for example, those disclosed in PCT Publications WO 93/08207 and WO 94/04570 and European Patent Publication Nos. 555 880 A2 and 585 943 A2).

When a function associated with the CD40 pathway should be augmented, nucleic acid molecules coding for CD40bp can be inserted into a CD40<sup>+</sup> cell, such as a B cell, using an appropriate pharmaceutical vector. Alternatively, when a function, associated with the CD40  
30- pathway should be suppressed a nucleic acid coding for CD40bp fragment, a dominant inhibitory CD40bp polypeptide fragment, or anti-sense CD40bp RNA can be introduced into a CD40<sup>+</sup> cell using an appropriate pharmaceutical vector.

This method can be practiced *in vitro*, *ex vivo*  
35 or *in vivo*. When the method is practiced *in vitro* or *ex vivo*, the expression vector, protein or polypeptide can

-27-

be added to the cells in culture or taken from a subject or added to a pharmaceutically acceptable carrier as defined below. In addition, the expression vector or CD40bp DNA can be inserted into the target cell using well known techniques such as transfection, electroporation or microinjection.

More specifically, the *in vitro* assay method comprises culturing suitable cell cultures or tissue cultures under conditions (temperature, growth or culture medium and gas (CO<sub>2</sub>)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. Suitable cell or tissue cultures or CD40<sup>+</sup> or CD40<sup>-</sup>. In one embodiment, the cells are then exposed to preliminary conditions for CD40 activation, e.g., by exposing them to CD40L or to CD40 antibodies such as those described in Armitage et al. (1992) supra, Armitage et al. (1993) supra, Caux et al. (1994) supra, Torres and Clark (1992) supra, or Werner-Favre et al. (1994) supra. CD40L and CD40 antibodies are well known to those of skill in the art. (See PCT Publication Nos. WO 93/08207<sup>supra</sup> and 94/04570<sup>supra</sup>). The "activated" cells are again cultured under suitable temperature and time conditions. In some embodiments, a drug or agent to be tested is added in varying concentrations at a time that is simultaneous with, prior to, or after the activating agent.

The nucleic acid or protein of this invention is then added to the culture in an effective amount and the cells are cultured under suitable temperature and time conditions to effect transcription of the nucleic acid or binding of the protein to the receptor. The nucleic acid or protein can be added prior to, simultaneously with, or after, the activating agent. The cells are assayed for CD40 activity using methods well known to those of skill in the art and described herein, for example, by monitoring CD40-associated IgG

-28-

production. It is apparent to those of skill in the art that two separate culture of cells must be treated and maintained as the test population. One is maintained without receiving an activating agent to determine  
5 background release and the second without receiving the agent to be tested. This second population of cells acts as a control.

When the method is practiced *in vivo* in a human patient or when activated cells are treated *ex vivo*, it  
10 is unnecessary to provide the activating agent since it is provided by the patient's immune system. However, when practiced in an experimental animal model, it can be necessary to provide an effective amount of the activating agent in a pharmaceutically acceptable carrier  
15 prior to administration of the nucleic acid or protein to activate CD40<sup>+</sup> cells. When the method is practiced *in vivo*, the carrying vector, polypeptide, polypeptide equivalent, or expression vector can be added to a pharmaceutically acceptable carrier and systemically  
20 administered to the subject, such as a human patient or an animal such as a mouse, a guinea pig, a simian, a rabbit or a rat. Alternatively, it can be directly infused into the cell by microinjection.

When practiced *in vivo*, the compositions and  
25 methods are particularly useful for maintaining CD40 function in a subject or an individual suffering from or predisposed to suffer from CD40-related disfunction, such as Hyper-IgM Syndrome. When the animal is an experimental animal such as a mouse, this method provides  
30 a powerful assay to screen for new drugs that may be used alone or in combination with this invention to ameliorate or reduce the symptoms and infections associated with CD40-related disfunction.

As used herein, the term "administering" for *in vivo* purposes means providing the subject with an  
35 effective amount of the nucleic acid molecule,

polypeptide or antibody, effective to modulate CD40-related function of the target cell. Methods of administering pharmaceutical compositions are well known to those of skill in the art and include, but are not  
5 limited to, microinjection, intravenous or parenteral administration. The compositions are intended for topical, oral, or local administration as well as intravenously, subcutaneously, or intramuscularly. Administration can be effected continuously or  
10 intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the vector used for therapy, the polypeptide or protein used for therapy, the  
15 purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician.

The compositions also can be administered to  
20 subjects or individuals susceptible to or at risk of developing a CD40-related disease. In one embodiment, the composition can be administered to a subject susceptible to CD40-related lymphocyte disfunction to maintain lymphocyte cell function such as antibody  
25 production. In these instances, a "prophylactically effective amount" of the composition is administered which is defined herein to be an amount that is effective to maintain the targeted CD40 function, such as lymphocyte function, at an acceptable level.

30 It should be understood that by preventing or inhibiting CD40 disfunction in a subject or individual, the compositions and methods of this invention also provide methods for treating, preventing or ameliorating the symptoms associated with a disease characterized by  
35 CD40 disfunction.

The following examples are provided merely to illustrate, but not limit, the invention described herein.

### Experiment I

5

Yeast Two-hybrid Screen -- Using a modification of the method of Harper et al. (1993) Cell 75:805-816, a hybrid gene encoding the GAL4 DNA-binding domain (amino acids 1-147), hemagglutinin ("HA") epitope tag, and CD40 cytoplasmic region (amino acids 216-279) was constructed in the yeast bait vector pAS1CYH2. This construct was designated GAL4CD40, and expression of the fusion protein was confirmed by anti-HA immunoblotting. This bait plasmid was cotransformed with a human B-cell cDNA expression library (prey) fused to the activation domain of GAL4 in the pACT plasmid. Interaction between bait- and prey-encoded genes in the Y190 yeast strain reconstitutes GAL4 as an active transcriptional complex, allowing growth in the absence of histidine and activation of the  $\beta$ -galactosidase reporter gene. Thirty-six of the  $10^6$  transformants screened grew in the absence of histidine and had detectable  $\beta$ -galactosidase staining within 10 minutes of incubation with the chromogenic substrate 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside. Plasmids recovered from the original yeast strains were used in a cotransformation assay with GAL4CD40 or control heterologous baits. Twelve plasmids encoded proteins that interacted with native CD40 but not with the control heterologous baits. DNA sequencing revealed 9 of 12 to encode the same protein, designated CD40-binding protein (CD40bp). Cotransformation assays were repeated in the yeast Y190 strain, where CD40bp fused to the activation domain of GAL4 was cotransformed with native CD40 (pCD40T) or the indicated heterologous baits expressed as fusions with the DNA-binding domain of GAL4. These included mutant CD40 (where Thr<sup>234</sup> was changed to an

alanine: pCD40A), the cytoplasmic domain of the p55 TNF receptor (pTNFR amino acids 206-426), FAS receptor cytoplasmic domain (pFAS amino acids 178-319), truncated p55 TNF receptor cytoplasmic domain missing 20 C-terminal residues (ΔTNFR amino acids 206-406), the helix-loop-helix motif of E12 (amino acids 508-564) (from Staudinger et al. (1993) J. Biol. Chem. 268:4608-4611) and the yeast Ser-Thr kinase SNF1 (from Yang et al. (1992) Science 257:680-682). Colonies from each transformation were patched onto a selective plate and a β-galactosidase assay performed on yeast transferred to nitrocellulose filters and permeabilized in liquid nitrogen as described in Harper et al. (1993) supra.

Nine independent clones were found to encode the same protein, designated CD40-binding protein (CD40bp) in the yeast Y190 strain (Figure 1). To assess whether the interaction of CD40bp was specific to native CD40, a mutant CD40 bait was created in which Thr<sup>234</sup> was converted to Ala (pCD40A), an alteration known to disable CD40 signaling. In addition, other heterologous baits, including the cytoplasmic domains of the related TNF and FAS receptors, were tested in a cotransformation assay. As shown in Figure 1, CD40bp interacted with native CD40 only but not with mutant CD40 or the other heterologous baits, showing that the CD40-CD40bp interaction was specific as measured by the yeast cotransformation assay.

## Experiment II

*GST Fusion Protein Expression and In Vitro Binding Assay* -- Native (CD40T) and mutant (CD40A) CD40 sequences used in the construction of the yeast bait vectors were excised and subcloned into the glutathione S-transferase ("GST") fusion protein vector pGStag (as described in Ron et al. (1992) BioTechniques 13:866-869) and transformed into the *Escherichia coli* strain BL21

-32-

(DF3) pLyss (as described in Studier et al. (1991) J. Mol. Bio. 219:37-44). GST and GST fusion proteins were prepared using published procedures of Studier et al. (1991) supra, and the recombinant proteins were  
5 immobilized onto glutathione-agarose beads at a concentration of about 8 mg/ml (as described in Harper et al. (1993) supra).

Labeled CD40bp was prepared by *in vitro* transcription translation using the TNT T7-coupled  
10 reticulocyte lysate system from Promega according to the manufacturer's instructions. Briefly, a 2.2-kilobase pair cDNA encoding CD40bp was excised from the yeast prey vector (pACT) using *Xho*I and subcloned into the  
pBluescript II plasmid (Stratagene), which had a flanking  
15 T7 promoter allowing generation of sense strand transcript. The luciferase construct was provided by the vendor and could similarly be transcribed by T7 polymerase.

Following translation, 5  $\mu$ l of total  $^{36}$ S-  
20 labeled reticulocyte lysate was either subjected to SDS-polyacrylamide gel electrophoresis and fluorography or diluted into 1 ml of GST binding buffer (10 mM Tris, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride), and  
25 incubated with 40  $\mu$ l of a 50% slurry of GST-, GSTCD40T-, or GSTCD40A-agarose beads for 2 hours at 4°C, following which the beads were pelleted by pulse centrifugation in a microcentrifuge, washed three times in GST buffer  
(without bovine serum albumin), boiled in SDS-sample  
30 buffer, and resolved on a 10% SDS-acrylamide gel. Bound proteins were visualized following autoradiography at -80°C for 1 hour.

To independently confirm the CD40-CD40bp interaction, the identical cytoplasmic domain regions of  
35 CD40 and mutant CD40 used in the yeast two-hybrid system were expressed as GST fusion proteins, immobilized to



glutathione-agarose beads, and used to precipitate radiolabeled *in vitro* translated CD40bp. Figure 2 shows *in vitro* translated CD40bp migrating with an apparent molecular mass of 64 kDa, which closely approximates the predicted molecular mass. CD40bp was effectively precipitated by native CD40 (GSTCD40T) but not by GST alone or, more significantly, by mutant CD40 (GSTCD40A). Furthermore, none of the GST proteins precipitated luciferase, a control for nonspecific binding. These studies further prove the specificity of the CD40-CD40bp interaction and implicate Thr<sup>234</sup> in the CD40 cytoplasmic domain as being fundamentally important in both signaling and CD40bp binding.

15

## Experiment III

*Construction of CD40 and CD40bp Expression*

Vectors -- Full-length CD40 coding sequence was obtained by PCR from a human B-cell library (as described in Harper et al. (1993) *supra*) and confirmed by sequencing. The primers used were: CGGGGTACCGCCACCATGG-TTCGTCTGCCTCTGCAG for the upstream primer and TTTGTCGAC-TCACTGTCTCTCCTGCAC for the downstream primer. The upstream primer had a built-in *Kpn*I site and the downstream primer a *Sal*I site (underlined) to facilitate cloning into the eukaryotic expression vector pcDNA3 (Invitrogen). Mutant CD40 (pCD40A) was made by site-directed mutagenesis using a two-step PCR protocol of Higuchi et al. (1988) *Nucleic Acids Res.* 16:7351-7367, and employing two additional oligonucleotides: GCTCCAG-TGCAGGAAGCTTTACATGGATGC and GCATCCATGTAAAGCTTCCTGCACTGG-AGC (altered bases are underlined). The Thr<sup>234</sup> → Ala mutation in pCD40A was confirmed by sequence analysis.

To construct pHATagCD40bp, CD40bp was excised from the yeast vector pACT by *Xho*I digestion and subcloned into pcDNA3 in which an HA epitope tag

4  
(SER 1) 10:14  
(YPYDVPDYA) had previously been placed downstream of the cytomegalovirus promoter/enhancer. The orientation of CD40bp and the junctional sequence between the HA tag and CD40bp were confirmed by sequence analysis.

5 To demonstrate the interaction in vivo, 293T cells, a human epithelioid cell line available from the ATCC, (which is CD40-negative), were cotransfected with a HA epitope-tagged CD40bp expression construct and vector alone, mutant CD40 (CD40A), or native CD40 (CD40T)  
10 expression constructs. Following metabolic labeling with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, cell lysates were subjected to an immunoprecipitation analysis with an anti-CD40 monoclonal antibody (Figure 3A). No labeled protein was immunoprecipitated in vector-transfected  
15 cells, while, as expected, CD40 was immunoprecipitated in both CD40A and CD40T transfectant. However, only in cells transfected with native CD40 (CD40T) was there a co-precipitating protein whose molecular size corresponded to CD40bp. To confirm the identity of the  
20 precipitating proteins in the CD40T-transfected cells, the immune complex was dissociated and subjected to a second round of immunoprecipitation, as shown in Figure 3B, with control anti-thrombospondin ( $\alpha$ -TSP) antibody, anti-CD40 monoclonal antibody, or anti-HA epitope tag  
25 antibody (to identify HA-tagged CD40bp). While no labeled protein was precipitated by the control antibody, the anti-CD40 and anti-HA tag antibodies confirmed the presence of CD40 and CD40bp in the original immune complex.

30

## Experiment IV

*Transfection, Metabolic Cell Labeling, and Immunoprecipitation Analysis* -- These methods were performed essentially as described in O'Rourke et al. (1992) J. Biol. Chem. 267:24921-24924. For re-immunoprecipitation analysis, the initial immune complex was dissociated by boiling in PBS + 1% SDS, diluted 10-fold in PBS containing 1% Triton X-100 and 1% deoxycholate, and subjected to a second round of immunoprecipitation analysis.

To conclusively show that CD40bp interacted with native CD40 in B-cells, the Epstein-Barr virus-negative, CD40-positive human B-cell line BJAB was transiently transfected with the epitope-tagged CD40bp expression construct and metabolically labeled, and endogenous CD40 was immunoprecipitated with an anti-CD40 monoclonal antibody (Figure 3C). Autoradiographic analysis of the precipitated proteins following SDS-polyacrylamide gel electrophoresis revealed, as expected, the presence of CD40 receptor but also that of two associated proteins, one that migrated just larger than CD40bp( $\Delta$ ) and a fainter band that migrated at the expected molecular weight for CD40bp(\*). To confirm that this was indeed CD40bp, the immune complex was dissociated and subjected to a second round of immunoprecipitation with either anti-HA epitope tag antibody or isotype-matched control antibody. CD40bp (corresponding to the band marked by an asterisk) was clearly immunoprecipitated by the anti-HA antibody and not by control antibody. This confirmed the presence of CD40bp in the original anti-CD40 immune complex and indicated that this insertion was capable of occurring in B-cells. Expression of CD40bp transcript in B-cell lines was confirmed by Northern blot and RT-PCR analysis (Figures 3D and 3E).

## Experiment V

Transcript Analysis -- mRNA analysis by Northern blotting and reverse transcriptase PCR ("RT-PCR") was performed as described previously in O'Rourke et al. (1992) supra using a commercially available kit from Perkin-Elmer. For the Northern blot, 7  $\mu$ g of poly(A)<sup>+</sup> RNA from SKW6.4 cells was hybridized to a <sup>32</sup>P-labeled CD40bp encoding XhoI fragment.

For RT-PCR, CD40bp-specific 18-mer oligonucleotide primers were used. The downstream primer (AGAGGAGTTGCCTTCTGC) was used initially for the reverse transcriptase reaction and later for PCR in conjunction with an upstream primer (GGCATGACCAGATGCTGA) to give an expected size product of ~600 base pairs on agarose gel electrophoresis.

DNA Sequencing and Data Base Searching -- Double stranded plasmid template was sequenced on both strands as described previously in O'Rourke et al. (1992) supra using modified T7 DNA polymerase. Initial data base homology searching revealed hundreds of matches to myosins and other  $\alpha$ -helical, coiled-coil proteins. To further characterize portions of the CD40bp that might have coiled-coil potential, we used the COILS 2 program of Lupas et al. ((1991) Science 252:1162-1164), which has been updated recently (at lupas@ums.biochem.mpg.cle).

The deduced sequence of the 2350-base pair CD40bp cDNA revealed an open reading frame that began with an initiator methionine conforming to Kozak's consensus and that ended 567 residues later at an Opal codon. Given the presence of the open reading frame and the size of the CD40bp transcript (~2.5 kilobase pairs; Figure 3D), it is likely that Figure 4A represents the full-length coding sequence. Homology searching and use of the COILS algorithm revealed a discrete coiled-coil domain spanning residues 266-366 and flanked by regions

without coiled-coil potential (Figure 4D). Residues 266-366 of CD40bp were then "masked" by the method of Altschul et al. (1994) Nature Genet. 6:119-129, and the database searches repeated. In this case there were 12 statistically significant ( $p < 0.05$ ) matches, all to proteins known to contain the "RING finger" DNA-binding motif. Six of the 12 matches (including the most significant match) were to V(D)J recombination activating proteins (RAD1) from various species.

10                   Importantly, one of the matches was the N-terminal RING finger sequence motif of TRAF2, which together with TRAF1, binds to the cytoplasmic domain of the 75-kDa TNF receptor as a heterodimeric complex in which TRAF2 contacts the receptor directly. The  
15                   remaining matches included the human RING 1 gene product itself, the 52-kDa ribonucleoprotein autoantigen in Sjogren's syndrome, the *Neurospora uvs-2* gene product thought to be involved in DNA repair, and a developmentally regulated *Dictyostelium* gene (DG17) of  
20                   unknown function. The region between the RING finger and coiled-coil domains contains 17 cysteines and 10 histidines out of a total of 168 residues. These Cys/His residues are arranged in patterns resembling the "B box" motifs observed in some other RING finger proteins.  
25                   Neither the RING finger or the coiled-coil segment, a motif known to mediate homo- and/or hetero-oligomerization appears necessary for binding to CD40 since one class of interacting CD40bp cDNAs identified in the two-hybrid screen encoded only the C-terminal half of  
30                   CD40bp (beginning at Phe<sup>297</sup>, which deletes the RING finger and truncates the coiled-coil segment). Instead, it appears likely that the C-terminal portion mediates CD40 binding.

                  This is supported by the finding that a  
35                   similarly truncated TRAF2 protein (missing the RING finger domain) could still associate with the 75-kDa TNF

-38-

receptor. In keeping with a common function for the C termini of these proteins is the remarkable sequence similarity that exists between the C-terminal half of CD40bp and the TRAF domains of TRAF1 and TRAF2 (Figure 4E). Except for the RING finger domain in TRAF2, the three molecules are fairly distinct at their N-terminal halves. Taken together, these studies suggest that existence of a new family of proteins that associate with the cytoplasmic faces of the TNF receptor family and have in common the TRAF domain. Finally, given that TRAF1 and TRAF2 also possess central coiled-coil motifs, it will be important to determine if CD40bp can heterodimerize with these proteins.

Throughout this application, reference is made to various journal articles, U.S. patents and published applications. The disclosures of these references are hereby incorporated by reference into the present disclosure.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: DIXIT, VISHA M.
- (ii) TITLE OF INVENTION: CD40 BINDING COMPOSITIONS AND METHODS OF USING SAME
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: MORRISON & FOERSTER  
(B) STREET: 755 Page Mill Road  
(C) CITY: Palo Alto  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: US  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: KONSKI, ANTOINETTE F.  
(B) REGISTRATION NUMBER: 34,202  
(C) REFERENCE/DOCKET NUMBER: 203442102500
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (415) 813-5600  
(B) TELEFAX: (415) 494-0792  
(C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 2339 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 211..1911

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACGAAGGCCA CGCGCCCGGC GCCCCTGAGC CGGCCGAGCG GCGACGGACC GCGAGATGAG	60
GAAAATGAGG CCCAAAGAAG TGATGCCACT.TGGTTAAGGT CCCAGAGCAG GTCAGAATCA	120
GACCTAGGAT CAGAAACCTG GCTCCTGGCT CCTGCTCCT ACTCTTCTAA GGATCGCTGT	180
CCTGACAGAA GAGAACTCCT CTTTCCTAAA ATG GAG TCG AGT AAA AAG ATG GAC	234
Met Glu Ser Ser Lys Lys Met Asp	

5

GCC CAC GAG GCC AGC TCC GCC GTG CAG CAC GTC AAC CTG CTG AAG GAG 1002



Ala	His	Glu	Ala	Ser	Ser	Ala	Val	Gln	His	Val	Asn	Leu	Leu	Lys	Glu	
250						255					260					
TGG	AGC	AAC	TCG	CTC	GAA	AAG	AAG	GTT	TCC	TTG	TTG	CAG	AAT	GAA	AGT	1050
Trp	Ser	Asn	Ser	Leu	Glu	Lys	Lys	Val	Ser	Leu	Leu	Gln	Asn	Glu	Ser	
265					270					275					280	
GTA	GAA	AAA	AAC	AAG	AGC	ATA	CAA	AGT	TTG	CAC	AAT	CAG	ATA	TGT	AGC	1098
Val	Glu	Lys	Asn	Lys	Ser	Ile	Gln	Ser	Leu	His	Asn	Gln	Ile	Cys	Ser	
				285					290					295		
TTT	GAA	ATT	GAA	ATT	GAG	AGA	CAA	AAG	GAA	ATG	CTT	CGA	AAT	AAT	GAA	1146
Phe	Glu	Ile		Ile	Glu	Arg	Gln	Lys	Glu	Met	Leu	Arg	Asn	Asn	Glu	
			300					305					310			
TCC	AAA	ATC	CTT	CAT	TTA	CAG	CGA	GTG	ATA	GAC	AGC	CAA	GCA	GAG	AAA	1194
Ser	Lys	Ile	Leu	His	Leu	Gln	Arg	Val	Ile	Asp	Ser	Gln	Ala	Glu	Lys	
		315					320					325				
CTG	AAG	GAG	CTT	GAC	AAG	GAG	ATC	CGG	CCC	TTC	CGG	CAG	AAC	TGG	GAG	1242
Leu	Lys	Glu	Leu	Asp	Lys	Glu	Ile	Arg	Pro	Phe	Arg	Gln	Asn	Trp	Glu	
	330					335				340						
GAA	GCA	GAC	AGC	ATG	AAG	AGC	AGC	GTG	GAG	TCC	CTC	CAG	AAC	CGC	GTG	1290
Glu	Ala	Asp	Ser	Met	Lys	Ser	Ser	Val	Glu	Ser	Leu	Gln	Asn	Arg	Val	
345					350					355					360	
ACC	GAG	CTG	GAG	AGC	GTG	GAC	AAG	AGC	GCG	GGG	CAA	GTG	GCT	CGG	AAC	1338
Thr	Glu	Leu	Glu	Ser	Val	Asp	Lys	Ser	Ala	Gly	Gln	Val	Ala	Arg	Asn	
				365					370					375		
ACA	GGC	CTG	CTG	GAG	TCC	CAG	CTG	AGC	CGG	CAT	GAC	CAG	ATG	CTG	AGT	1386
Thr	Gly	Leu	Leu	Glu	Ser	Gln	Leu	Ser	Arg	His	Asp	Gln	Met	Leu	Ser	
			380					385					390			
GTG	CAC	GAC	ATC	CGC	CTA	GCC	GAC	ATG	GAC	CTG	GGC	TTC	CAG	GTC	CTG	1434
Val	His	Asp	Ile	Arg	Leu	Ala	Asp	Met	Asp	Leu	Gly	Phe	Gln	Val	Leu	
		395					400					405				
GAG	ACC	GCC	AGC	TAC	AAT	GGA	GTG	CTC	ATC	TGG	AAG	ATT	CGC	GAC	TAC	1482
Glu	Thr	Ala	Ser	Tyr	Asn	Gly	Val	Leu	Ile	Trp	Lys	Ile	Arg	Asp	Tyr	
	410					415					420					
AAG	CGG	CGG	AAG	CAG	GAG	GCC	GTC	ATG	GGG	AAG	ACC	CTG	TCC	CTT	TAC	1530
Lys	Arg	Arg	Lys	Gln	Glu	Ala	Val	Met	Gly	Lys	Thr	Leu	Ser	Leu	Tyr	
425					430					435					440	
AGC	CAG	CCT	TTC	TAC	ACT	GGT	TAC	TTT	GGC	TAT	AAG	ATG	TGT	GCC	AGG	1578
Ser	Gln	Pro	Phe	Tyr	Thr	Gly	Tyr	Phe	Gly	Tyr	Lys	Met	Cys	Ala	Arg	
				445					450					455		
GTC	TAC	CTG	AAC	GGG	GAC	GGG	ATG	GGG	AAG	GGG	ACG	CAC	TTG	TCG	CTG	

CGT CAT TTG GGA GAT GCA TTC AAG CCC GAC CCC AAC AGC AGC AGC TTC	1770
Arg His Leu Gly Asp Ala Phe Lys Pro Asp Pro Asn Ser Ser Ser Phe	
505 510 515 520	
AAG AAG CCC ACT GGA GAG ATG AAT ATC GCC TCT GGC TGC CCA GTC TTT	1818 =
Lys Lys Pro Thr Gly Glu Met Asn Ile Ala Ser Gly Cys Pro Val Phe	
525 530 535	
GTG GCC CAA ACT GTT CTA GAA AAT GGG ACA TAT ATT AAA GAT GAT ACA	1866
Val Ala Gln Thr Val Leu Glu Asn Gly Thr Tyr Ile Lys Asp Asp Thr	
540 545 550	
ATT TTT ATT AAA GTC ATA GTG GAT ACT TCG GAT CTG CCC GAT CCC	1911
Ile Phe Ile Lys Val Ile Val Asp Thr Ser Asp Leu Pro Asp Pro	
555 560 565	
TGATAAGTAG CTGGGGAGGT GGATTTAGCA GAAGGCAACT CCTCTGGGGG ATTTGAACCG	1971
GTCTGTCTTC ACTGAGGTCC TCGCGCTCAG AAAAGGACCT TGTGAGACGG AGGAAGCGGC	2031
AGAAGGCGGA CGCGTGCCGG CGGGAGGAGC CACGCGAGAG CACACCTGAC ACGTTTTATA	2091
ATAGACTAGC CACACTTCAC TCTGAAGAAT TATTTATCCT TCAACAAGAT AAATATTGCT	2151
GTCAGAGAAG GTTTTCATTT TCATTTTAA AGATCTAGTT AATTAAGGTG GAAAACATAT	2211
ATGCTAAACA AAAGAAACAT GATTTTCTT CCTTAACTT GAACACCAA AAAACACACA	2271
CACACACACA CGTGGGGATA GCTGGACATG TCAGCATGTT AAGTAAAAGG AGAATTTATG	2331
AAATAGTA	2339

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Glu	Ser	Ser	Lys	Lys	Met	Asp	Ser	Pro	Gly	Ala	Leu	Gln	Thr	Asn
1				5					10					15	
Pro	Pro	Leu	Lys	Leu	His	Thr	Asp	Arg	Ser	Ala	Gly	Thr	Pro	Val	Phe
			20					25					30		
Val	Pro	Glu	Gln	Gly	Gly	Tyr	Lys	Glu	Lys	Phe	Val	Lys	Thr	Val	Glu
		35					40					45			
Asp	Lys	Tyr	Lys	Cys	Glu	Lys	Cys	His	Leu	Val	Leu	Cys	Ser	Pro	Lys
	50					55					60				
Gln	Thr	Glu	Cys	Gly	His	Arg	Phe	Cys	Glu	Ser	Cys	Met	Ala	Ala	Leu
	65				70					75					80
Leu	Ser	Ser	Ser	Ser	Pro	Lys	Cys	Thr	Ala	Cys	Gln	Glu	Ser	Ile	Val
				85					90					95	
Lys	Asp	Lys	Val	Phe	Lys	Asp	Asn	Cys	Cys	Lys	Arg	Glu	Ile	Leu	Ala
			100					105					110		

Leu	Gln	Ile	Tyr	Cys	Arg	Asn	Glu	Ser	Arg	Gly	Cys	Ala	Glu	Gln	Leu
		115					120					125			
Met	Leu	Gly	His	Leu	Val	His	Leu	Lys	Asn	Asp	Cys	His	Phe	Glu	Glu
	130					135					140				
Leu	Pro	Cys	Val	Arg	Pro	Asp	Cys	Lys	Glu	Lys	Val	Leu	Arg	Lys	Asp
145					150					155					160
Leu	Arg	Asp	His	Val	Glu	Lys	Ala	Cys	Lys	Tyr	Arg	Glu	Ala	Thr	Cys
				165					170					175	
Ser	His	Cys	Lys	Ser	Gln	Val	Pro	Met	Ile	Ala	Leu	Gln	Lys	His	Glu
			180					185					190		
Asp	Thr	Asp	Cys	Pro	Cys	Val	Val	Val	Ser	Cys	Pro	His	Lys	Cys	Ser
		195					200					205			
Val	Gln	Thr	Leu	Leu	Arg	Ser	Glu	Leu	Ser	Ala	His	Leu	Ser	Glu	Cys
	210					215					220				
Val	Asn	Ala	Pro	Ser	Thr	Cys	Ser	Phe	Lys	Arg	Tyr	Gly	Cys	Val	Phe
225					230					235					240
Gln	Gly	Thr	Asn	Gln	Gln	Ile	Lys	Ala	His	Glu	Ala	Ser	Ser	Ala	Val
			245						250					255	
Gln	His	Val	Asn	Leu	Leu	Lys	Glu	Trp	Ser	Asn	Ser	Leu	Glu	Lys	Lys
			260					265					270		
Val	Ser	Leu	Leu	Gln	Asn	Glu	Ser	Val	Glu	Lys	Asn	Lys	Ser	Ile	Gln
		275					280					285			
Ser	Leu	His	Asn	Gln	Ile	Cys	Ser	Phe	Glu	Ile	Glu	Ile	Glu	Arg	Gln
	290					295					300				
Lys	Glu	Met	Leu	Arg	Asn	Asn	Glu	Ser	Lys	Ile	Leu	His	Leu	Gln	Arg
305					310					315					320
Val	Ile	Asp	Ser	Gln	Ala	Glu	Lys	Leu	Lys	Glu	Leu	Asp	Lys	Glu	Ile
				325					330					335	
Arg	Pro	Phe	Arg	Gln	Asn	Trp	Glu	Glu	Ala	Asp	Ser	Met	Lys	Ser	Ser
			340					345					350		
Val	Glu	Ser	Leu	Gln	Asn	Arg	Val	Thr	Glu	Leu	Glu	Ser	Val	Asp	Lys
		355					360					365			
Ser	Ala	Gly	Gln	Val	Ala	Arg	Asn	Thr	Gly	Leu	Leu	Glu	Ser	Gln	Leu
	370					375					380				
Ser	Arg	His	Asp	Gln	Met	Leu	Ser	Val	His	Asp	Ile	Arg	Leu	Ala	Asp
385					390					395					400
Met	Asp	Leu	Gly	Phe	Gln	Val	Leu	Glu	Thr	Ala	Ser	Tyr	Asn	Gly	Val
				405					410					415	
Leu	Ile	Trp	Lys	Ile	Arg	Asp	Tyr	Lys	Arg	Arg	Lys	Gln	Glu	Ala	Val
			420					425					430		
Met	Gly	Lys	Thr	Leu	Ser	Leu	Tyr	Ser	Gln	Pro	Phe	Tyr	Thr	Gly	Tyr
	435						440					445			

Phe Gly Tyr Lys Met Cys Ala Arg Val Tyr Leu Asn Gly Asp Gly Met  
 450 455 460

Gly Lys Gly Thr His Leu Ser Leu Phe Phe Val Ile Met Arg Gly Glu  
 465 470 475 480

Tyr Asp Ala Leu Leu Pro Trp Pro Phe Lys Gln Lys Val Thr Leu Met  
 485 490 495

Leu Met Asp Gln Gly Ser Ser Arg Arg His Leu Gly Asp Ala Phe Lys  
 500 505 510

Pro Asp Pro Asn Ser Ser Ser Phe Lys Lys Pro Thr Gly Glu Met Asn  
 515 520 525

Ile Ala Ser Gly Cys Pro Val Phe Val Ala Gln Thr Val Leu Glu Asn  
 530 535 540

Gly Thr Tyr Ile Lys Asp Asp Thr Ile Phe Ile Lys Val Ile Val Asp  
 545 550 555 560

Thr Ser Asp Leu Pro Asp Pro  
 565

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 49 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Lys Tyr Lys Cys Glu Lys Cys His Leu Val Leu Cys Ser Pro Lys  
 1 5 10 15

Gln Thr Glu Cys Gly His Arg Phe Cys Glu Ser Cys Met Ala Ala Leu  
 20 25 30

Leu Ser Ser Ser Ser Pro Lys Cys Thr Ala Cys Gln Glu Ser Ile Val  
 35 40 45

Lys

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 50 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Lys Tyr Leu Cys Ser Ala Cys Lys Asn Ile Leu Arg Arg Pro Phe  
 1 5 10 15

Gln Ala Gln Cys Gly His Arg Tyr Cys Ser Phe Cys Leu Thr Ser Ile  
 20 25 30

Leu Ser Ser Gly Pro Gln Asn Cys Ala Ala Cys Val Tyr Glu Gly Leu  
 35 40 45  
 Tyr Glu  
 50

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Ser Ile Ser Cys Gln Ile Cys Glu His Ile Leu Ala Asp Pro Val  
 1 5 10 15  
 Glu Thr Asn Cys Lys His Val Phe Cys Arg Val Cys Ile Leu Arg Cys  
 20 25 30  
 Leu Lys Val Met Gly Ser Tyr Cys Pro Ser Cys Arg Tyr Pro Cys Phe  
 35 40 45  
 Pro

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 50 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Glu Leu Met Cys Pro Ile Cys Leu Asp Met Leu Lys Asn Thr Met  
 1 5 10 15  
 Thr Thr Lys Glu Cys Leu His Arg Phe Cys Ser Asp Cys Ile Val Thr  
 20 25 30  
 Ala Leu Arg Ser Gly Asn Lys Glu Cys Pro Thr Cys Arg Lys Lys Leu  
 35 40 45  
 Val Ser  
 50

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

Gln Ala Phe Arg Cys His Val Cys Lys Asp Phe Tyr Asp Ser Pro Met  
1 5 10 15  
Leu Thr Ser Cys Asn His Thr Phe Cys Ser Leu Cys Ile Arg Arg Cys  
20 25 30  
Leu Ser Val Asp Ser Lys Cys Pro Leu Cys Arg Ala Thr Asp Gln Glu  
35 40 45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 179 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln Met Leu Ser Val His Asp Ile Arg Leu Ala Asp Met Asp Leu Gly  
1 5 10 15  
Phe Gln Val Leu Glu Thr Ala Ser Tyr Asn Gly Val Leu Ile Trp Lys  
20 25 30  
Ile Arg Asp Tyr Lys Arg Arg Lys Gln Glu Ala Val Met Gly Lys Thr  
35 40 45  
Leu Ser Leu Tyr Ser Gln Pro Phe Tyr Thr Gly Tyr Phe Gly Tyr Lys  
50 55 60  
Met Cys Ala Arg Val Tyr Leu Asn Gly Asp Gly Met Gly Lys Gly Thr  
65 70 75 80  
His Leu Ser Leu Phe Phe Val Ile Met Arg Gly Glu Tyr Asp Ala Leu  
85 90 95  
Leu Pro Trp Pro Phe Lys Gln Lys Val Thr Leu Met Leu Met Asp Gln  
100 105 110  
Gly Ser Ser Arg Arg His Leu Gly Asp Ala Phe Lys Pro Asp Pro Asn  
115 120 125  
Ser Ser Ser Phe Lys Lys Pro Thr Gly Glu Met Asn Ile Ala Ser Gly  
130 135 140  
Cys Pro Val Phe Val Ala Gln Thr Val Leu Glu Asn Gly Thr Tyr Ile  
145 150 155 160  
Lys Asp Asp Thr Ile Phe Ile Lys Val Ile Val Asp Thr Ser Asp Leu  
165 170 175  
Pro Asp Pro

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 176 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Ser Ile Gly Leu Lys Asp Leu Ala Met Ala Asp Leu Glu Gln Lys  
1 5 10 15  
Val Ser Glu Leu Glu Val Ser Thr Tyr Asp Gly Val Phe Ile Trp Lys  
20 25 30  
Ile Ser Asp Phe Thr Arg Lys Arg Gln Glu Ala Val Ala Gly Arg Thr  
35 40 45  
Pro Ala Ile Phe Ser Pro Ala Phe Tyr Thr Ser Arg Tyr Gly Tyr Lys  
50 55 60

(2) INFORMATION FOR SEQ ID NO:12:

(A) LENGTH: 176 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

Gln 1	Thr	Leu	Ala 5	Gln	Lys	Asp	Gln	Val 10	Leu	Gly	Lys	Leu	Glu	His 15	Ser
Leu	Arg	Leu	Met 20	Glu	Glu	Ala	Ser	Phe 25	Asp	Gly	Thr	Phe	Leu 30	Trp	Lys
Ile	Thr 35	Asn	Val	Thr	Lys	Arg	Cys 40	His	Glu	Ser	Val	Cys 45	Gly	Arg	Thr
Val 50	Ser	Leu	Phe	Ser	Pro	Ala 55	Phe	Tyr	Thr	Ala	Lys 60	Tyr	Gly	Tyr	Lys
Leu 65	Cys	Leu	Arg	Leu	Tyr 70	Leu	Asn	Gly	Asp	Gly 75	Ser	Gly	Lys	Lys	Thr 80
His	Leu	Ser	Leu 85	Phe	Ile	Val	Ile	Met 90	Arg	Gly	Glu	Tyr	Asp	Ala 95	Leu
Leu	Pro	Trp	Pro 100	Phe	Arg	Asn	Lys	Val 105	Thr	Phe	Met	Leu	Leu 110	Asp	Gln
Asn	Asn 115	Arg	Glu	His	Ala	Ile	Asp 120	Ala	Phe	Arg	Pro	Asp 125	Leu	Ser	Ser
Ala 130	Ser	Phe	Gln	Arg	Pro	Gln 135	Ser	Glu	Thr	Asn	Val 140	Ala	Ser	Gly	Cys
Pro 145	Leu	Phe	Phe	Pro	Leu 150	Ser	Lys	Leu	Gln	Ser 155	Pro	Lys	His	Ala	Tyr 160





**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**